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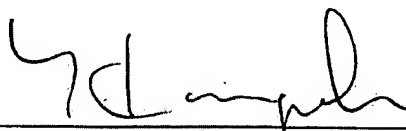
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Signed at Tokyo, Japan

This 17th day of July, 2008



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[Title of invention] Substance with antithrombic activity and method for detecting glyco-calicin

[Claims]

1. A method for detecting binding of von Willebrand factor and glycoprotein Ib or inhibition of the binding, comprising the steps of:

immobilizing von Willebrand factor in a reaction vessel in the presence of a substance inducing the binding of von Willebrand factor and glycoprotein Ib, and,

reacting the immobilized von Willebrand factor with glycoprotein Ib.

2. The method according to Claim 1, wherein the substance that induces the binding of von Willebrand factor and glycoprotein Ib is botrocetin.

3. A method for detecting binding of von Willebrand factor and glycoprotein Ib or inhibition of the binding, comprising the steps of:

binding a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ib α chain at its carboxyl terminus or the chimeric protein labeled with a labeling substance to von Willebrand factor immobilized in a reaction vessel, and

detecting the Fc region of the immunoglobulin molecule or the labeling substance.

4. The method according to Claim 3, wherein the substance that induces the binding of von Willebrand factor and glycoprotein Ib is botrocetin.

5. The method according to Claim 3, wherein von Willebrand factor is immobilized in the reaction vessel in the presence of a substance that induces the binding of von Willebrand factor and glycoprotein Ib.

6. The method according to Claim 1, wherein glyocalicin is measured by adding a sample containing glyocalicin to the reaction vessel when von Willebrand factor is reacted with glycoprotein Ib, or prior to the reacting, and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib.

7. The method according to Claim 3, wherein glyocalicin is measured by adding a sample containing glyocalicin to the reaction vessel when the chimeric protein is allowed to bind to von Willebrand factor, or prior to the binding, and detecting inhibition of the binding of von Willebrand factor and the chimeric protein.

8. The method according to Claim 1, wherein a substance that inhibits the binding of von Willebrand factor and glycoprotein Ib is detected by adding a sample containing a substance to be detected to the reaction vessel when von Willebrand factor is reacted with glycoprotein Ib, or prior to the reacting, and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib.

9. The method according to Claim 3, wherein a substance that inhibits the binding of von Willebrand factor and glycoprotein Ib is detected by adding a sample containing a substance to be

detected to the reaction vessel when the chimeric protein is allowed to bind to von Willebrand factor, or prior to the binding, and detecting inhibition of the binding of von Willebrand factor and the chimeric protein.

10. A chimeric protein, which consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ib α chain at its carboxyl terminus.

11. The chimeric protein according to Claim 10, wherein the immunoglobulin molecule is derived from mouse.

12. A kit for measuring glyco-calicin based on inhibition of a reaction of von Willebrand factor and glycoprotein Ib, comprising von Willebrand factor and a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ib α chain at its carboxyl terminus.

[Detailed description of the invention]

[0001]

[Technical field]

The present invention relates to substance with antithrombotic activity and method for detecting glyocalicin. More precisely, it relates to a method for detecting or measuring a substance that inhibits binding of von Willebrand factor and glycoprotein Ib, and means directly used for carrying out the method.

[0002]

[Background art]

The global number of patients with thromboses such as myocardial infarction, cerebral infarction and peripheral artery occlusive disease is very large, and these diseases are very significant diseases to be diagnosed and treated. Platelets play a fundamental role for the onset of these thromboses. In general, if vascular endothelial cells in blood vessel cavities are impaired by arteriosclerotic lesion or the like, platelets will adhere to the impaired region to cause activation, and thus there are formed thrombocytic thrombi, which eventually develop into occlusive lesions.

[0003]

As one of the methods for detecting activation of platelets, there is a method of measuring glyocalicin concentration in plasma. Glyocalicin is a protein consisting of an enzymatically cleaved extracellular portion of a membrane glycoprotein present on surfaces of platelets, glycoprotein Ib α chain, and has a molecular weight of about 135 kDa. It is known that glyocalicin concentration in plasma is increased by impairment or activation of platelets, and it is currently used as a marker for detecting presence or absence of thrombotic diseases

in clinical diagnosis. (J.H. Beer et al., *Blood*, 83, 691-702, 1994; S. Kunishima et al., *Clin. Chem.*, 37, 169-172, 1991).

[0004]

Many measurement methods of glyocalicin concentration have been reported, and any of these are based on ELISA (enzyme-linked immunosorbent assay) technique, wherein glyocalicin is detected by the sandwiching method utilizing two kinds of monoclonal antibodies directed to glyocalicin (J.H. Beer et al., *supra*; S. Kunishima et al., *supra*). Briefly, first monoclonal antibodies are immobilized on a 96-well plate or the like as a solid phase, blocked with a protein such as bovine serum albumin (BSA), and then added with patient's plasma (or serum) to be measured. Glyocalicin specifically binds to the monoclonal antibodies immobilized on the solid phase. The plate is washed, and added with second monoclonal antibodies labeled with an enzyme such as alkaline phosphatase and peroxidase or biotin so that the second antibodies should specifically bind to the glyocalicin bound to the first monoclonal antibodies. After washing, the plate is added with a substrate that can be converted into a substance exhibiting specific absorbance in a UV or visible region, fluorescence or luminescence with the enzyme used as the label of the second antibodies to perform an enzymatic reaction. Since the amount of glyocalicin in the patient's plasma and the binding amount of the second antibodies show positive correlation, the concentration of glyocalicin in the patient's plasma can be measured by quantifying the reaction product produced by the enzymatic reaction. A measurement method for glyocalicin by competitive ELISA utilizing one kind of anti-glyocalicin

antibodies has also been reported (H. Bessos et al., *Thromb. Res.*, 59, 497-507, 1990). However, the IC₅₀ value of the glyocalicin concentration showing competitive inhibition is about 4 µg/ml, and this makes the above measurement unusable for the measurement of the glyocalicin concentration in plasma (it is about 2 µg/ml in a healthy subject, J.H. Beer et al., supra).

[0005]

The aforementioned glyocalicin quantification methods based on the sandwich technique are widely used at present. However, when a similar measurement system is desired to be newly prepared, it is necessary to obtain two kinds of anti-glyocalicin monoclonal antibodies having different recognition sites. Commercially available monoclonal antibodies are generally very expensive, and the preparation of monoclonal antibodies requires much labor such as acquisition of glyocalicin for immunization, acquisition of hybridoma from a spleen of immunized mouse and screening of a monoclonal antibody-producing cell. Further, it is impossible to measure an absolute value of glyocalicin concentration from the amount of the enzymatic reaction in the aforementioned sandwich ELISA method, and in many cases, it is necessary to measure glyocalicin of several kinds of known concentrations to obtain a calibration curve, and then it is necessary to calculate a concentration in a test sample to be measured based on comparison with the calibration curve. Therefore, it is important to establish a method capable of measuring an absolute concentration of glyocalicin in a simple manner without the complicated preparation of monoclonal antibodies, from a viewpoint of wide use in clinical diagnosis.

[0006]

Further, in an early stage of onset of thrombosis, von Willebrand factor in blood binds to subendothelial tissues (collagen etc.) exposed due to impairment of vascular endothelial cells, and the membrane glycoprotein, glycoprotein Ib, on platelets binds to the von Willebrand factor. Thus, the platelets adhere to blood vessel walls, and they are activated (J.P. Cean et al., *J. Lab. Clin. Med.*, 87, 586-596, 1976; K.J. Clemetson et al., *Thromb. Haemost.*, 78, 266-270, 1997). Therefore, it is an important target of antithrombotic drugs for treating or preventing thromboses to inhibit the binding of von Willebrand factor and glycoprotein Ib. However, there are few substances that have been proven to exhibit antithrombotic property by inhibiting the binding of the both proteins.

[0007]

It has been reported that a recombinant protein VCL that has a sequence of from the 504th to 728th amino acid residues of von Willebrand factor shows an antithrombotic action by inhibiting the binding of von Willebrand factor and glycoprotein Ib (K. Azzam et al., *Thromb. Haemost.*, 73, 318-323, 1995). Further, it has also been reported that a monoclonal antibody AJvW-2 directed to human von Willebrand factor exhibits an antithrombotic activity by specifically binding to von Willebrand factor without showing hemorrhagic tendency (S. Kageyama et al., *Br. J. Pharmacol.*, 122, 165-171, 1997; WO 96/17078). Furthermore, the protein AS1051 derived from snake venom specifically binds to the platelet glycoprotein Ib to similarly exhibit an antithrombotic property without showing hemorrhagic tendency (N. Fukuchi et al., WO 95/08573).

Further, aurintricarboxylic acid, which is a

pigmental compound, has been reported to show an activity for inhibiting the binding of von Willebrand factor and glycoprotein Ib (M.D. Phillips et al., *Blood*, 72, 1989-1903, 1988). However, it is known that its binding specificity is not high (K. Azzam et al., *Thromb. Haemost.*, 75, 203-210, 1996; D. Mitra et al., *Immunology*, 87, 581-585, 1996; R.M. Lozano et al., *Eur. J. Biochem.*, 248, 30-36, 1997), and that the inhibition activity is exhibited by a polymerized macromolecule fraction (M. Weinstein et al., *Blood*, 78, 2291-2298, 1991; Z. Gual et al., *Thromb. Res.*, 71, 77-88, 1993; H. Matsuno et al., *Circulation*, 96, 1299-1304, 1997) etc.

[0008]

As described above, although it is an important target of antithrombotic drugs to inhibit the binding of von Willebrand factor and glycoprotein Ib, there is no low molecular weight compound that has reported to inhibit the binding of the both and have an antithrombotic activity, and therefore it is important to find out such a substance for attempting treatment and prevention of thromboses.

[0009]

The binding of von Willebrand factor and glycoprotein Ib is not observed under a usual condition, and it is considered that it occurs only under a condition where shear stress is induced in a blood flow (T.T. Vincent et al., *Blood*, 65, 823-831, 1985). However, as a method for artificially making it possible to observe the binding of the both proteins, there are known addition of an antibiotic, ristocetin (M.A. Howard and B.G. Firkin, *Thromb. Haemost.*, 26, 362-369, 1971), and addition of a protein derived from snake venom, botrocetin (M.S. Read et al., *Proc. Natl. Acad. Sci. USA.*, 75, 4514-4518, 1978). That is, the both substances are known

as a substance that binds to a specific site of von Willebrand factor to cause a structural change of the von Willebrand factor, thereby causing the binding of the von Willebrand factor and glycoprotein Ib, which does not occur under a usual condition. As a method for observing the binding of the both proteins, there is the following method reported by Fujimura et al. (Y. Fujimura et al., *Blood*, 77, 113-120, 1991).

[0010]

That is, human von Willebrand factor is labeled with ^{125}I in a conventional manner, and allowed to bind to formalin-fixed platelets in the presence of a certain amount of ristocetin or botrocetin. This binding occurs due to the specific binding of the von Willebrand factor to glycoprotein Ib on the surfaces of the immobilized platelets, and after unbound von Willebrand factor are removed by washing, the amount of the both proteins bound to each other can be measured by measuring the amount of ^{125}I . Miura et al. detected the binding of the both proteins by a similar method, wherein platelets were immobilized on a 96-well plate via immobilized anti-platelet membrane protein antibodies instead of the use of formalin-fixed platelets (S. Miura et al., *Anal. Biochem.*, 236, 215-220, 1996). Further, Matsui et al. reported a method of binding glycocalicin, which is a partial protein of the extracellular portion of glycoprotein Ib α chain in the presence of botrocetin, to von Willebrand factor bound to collagen immobilized as a solid phase (T. Matsui et al., *J. Biochem.*, 121, 376-381, 1997). Furthermore, Moriki et al. produced a recombinant protein expressing cell that expressed glycoprotein Ib on the membrane, and reported that ^{125}I -labeled von Willebrand factor bound to the glycoprotein Ib

on the membrane in the presence of botrocetin. Moriki et al. further produced a cell expressing glycoprotein Ib having a mutation in the amino acid sequence, which bound to von Willebrand factor without any inducing agent, and performed a binding experiment. However, the binding amount was very small compared with the binding amount in the presence of botrocetin or ristocetin (T. Moriki et al., *Blood*, 90, 698-705, 1997).

[0011]

As described above, all of the methods reported so far for detecting the binding of von Willebrand factor and glycoprotein Ib with high sensitivity without shearing force are exclusively methods utilizing addition of a binding inducing substance such as botrocetin or ristocetin to a liquid phase. However, the amount of botrocetin or ristocetin changes the amount of the binding of von Willebrand factor and glycoprotein Ib. Moreover, if a large number of binding experiments are performed by using a 96-well plate, for example, these methods utilizing addition of the inducing substance to the liquid phase are laborious. Furthermore, when the aforementioned low molecular weight substance inhibiting the binding of von Willebrand factor and glycoprotein Ib is searched, an extremely large number of binding experiments must be performed, and therefore it is necessary from this viewpoint to solve the aforementioned problem.

[0012]

[Problem to be solved by the invention]

The problems of the aforementioned technical background are summarized in the following three points.

[0013]

(1) Although methods for quantification of

glycocalicin is important for diagnosis of thromboses, conventional highly sensitive methods are sandwich ELISA methods. Therefore, two kinds of monoclonal antibodies having different recognition sites are required, and a calibration curve prepared with a standard substance is required for the quantification.

[0014]

(2) It is important to discover a low molecular weight inhibition substance for the binding of von Willebrand factor and glycoprotein Ib, and use it as a drug, in view of the treatment and prevention of thromboses. However, no low molecular weight drug has been known so far, which targets the inhibition of the binding of von Willebrand factor and glycoprotein Ib and is reported to have antithrombotic activity.

[0015]

(3) In order to find out such a drug as mentioned in the above (2), large number of binding inhibition experiments must be performed for von Willebrand factor and glycoprotein Ib. However, the methods comprising addition of a binding inducing substance to a liquid phase are complicated, and have problems concerning accuracy.

[0016]

The present invention has been accomplished from the aforementioned viewpoints, and an object of the present invention is to provide a method for detecting the binding of von Willebrand factor and glycoprotein Ib in a simple manner, a simple method for measurement of glycocalicin, and a simple method for measurement of a substance that can be an antithrombotic drug of which working point is the inhibition of the binding of von Willebrand factor and glycoprotein Ib, as well as means for use in

these measurement methods.

[0017]

[Means to solve the problem]

The inventors of the present invention assiduously studied in order to achieve the aforementioned object. That is, a protein expression system based on an animal cell was prepared first for obtaining a chimeric molecule consisting of a partial protein of glycoprotein Iba chain bound to the Fc region of immunoglobulin molecule (hereinafter referred to as "chimeric protein"). Further, they found that, if von Willebrand factor was immobilized in the presence of botrocetin, the aforementioned chimeric protein, i.e., glycoprotein Ib molecule, specifically bound to the immobilized von Willebrand factor without a binding inducing substance in a liquid phase, and that a binding test can be performed in a simple manner to measure the binding amount by labeling commercially available inexpensive anti-immunoglobulin Fc antibodies or directly labeling the chimeric protein, and thus accomplished the present invention.

[0018]

That is, the first method according to the present invention is a method for detecting binding of von Willebrand factor and glycoprotein Ib or inhibition of the binding, comprising the steps of immobilizing von Willebrand factor in a reaction vessel in the presence of a substance inducing the binding of von Willebrand factor and glycoprotein Ib, and, reacting the immobilized von Willebrand factor with glycoprotein Ib.

[0019]

The second method according to the present invention is a method for detecting binding of von

Willebrand factor and glycoprotein Ib or inhibition of the binding, comprising the steps of binding a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ib α chain at its carboxyl terminus or the chimeric protein labeled with a labeling substance to von Willebrand factor immobilized in a reaction vessel, and detecting the Fc region of the immunoglobulin molecule or the labeling substance.

[0020]

As the substance that induces the binding of von Willebrand factor and glycoprotein Ib, botrocetin can be mentioned.

Further, in another embodiment of the second method, von Willebrand factor is immobilized in the reaction vessel in the presence of a substance that induces the binding of von Willebrand factor and glycoprotein Ib.

[0021]

In the first and second methods, glyco-calicin contained in a sample can be measured by adding the sample to the reaction vessel during the reaction of von Willebrand factor and glycoprotein Ib, or prior to the reaction, and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib.

[0022]

Further, in the first and second methods, a substance that inhibits the binding of von Willebrand factor and glycoprotein Ib can be detected by adding a sample containing a substance to be detected to the reaction vessel during the reaction of von Willebrand factor and glycoprotein Ib, or prior to the reaction, and detecting inhibition of the binding of von Willebrand factor

and glycoprotein Ib.

[0023]

The present invention further provides a chimeric protein, which consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ib α chain at its carboxyl terminus.

[0024]

The present invention also provides a kit for measuring glyco-calicin based on inhibition of a reaction of von Willebrand factor and glycoprotein Ib, which comprises von Willebrand factor and a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ib α chain at its carboxyl terminus.

[0025]

The term "chimeric protein" used in the present specification means a chimeric protein that consists of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Ib at its carboxyl terminus. Further, the term "glycoprotein Ib" used for the methods of the present invention may refer to glycoprotein Ib itself or the chimeric protein, or the both of them.

The term "detection" used in the present specification mainly means finding out a substance or a phenomenon, but it may also mean measurement of amount of the substance or degree of the phenomenon as a result of the finding of the substance or the phenomenon. Further, the term "measurement" mainly means measurement of an amount of substance or a degree of phenomenon, but it may also mean finding

out the substance or the phenomenon.

[0026]

[Embodiments of the invention]

The present invention will be explained in detail hereinafter.

[0027]

<1> Chimeric protein

The chimeric protein of the present invention is a protein consisting of a partial protein comprising a von Willebrand factor binding site of glycoprotein Iba chain, which is one of platelet membrane proteins of human or other mammals, bound to an Fc region of a heavy chain (H chain) of immunoglobulin molecule of mouse, human or other mammals by means of a genetic engineering technique. This chimeric protein can be produced by using cultured cells. In the chimeric protein, the partial protein comprising von Willebrand factor binding site of glycoprotein Iba chain and the Fc region of the immunoglobulin molecule are bound at the carboxyl terminus of the partial protein and the amino terminus of the Fc region.

[0028]

As an example of the partial protein of glycoprotein Iba chain, there can be mentioned a partial protein having a sequence comprising amino acid residues of from the amino terminus to the aspartic acid residue at the 319th position (amino acid numbers 1-319) of glycoprotein Iba chain molecule. However, since it is considered that von Willebrand factor binding site is a region contained in the amino acid sequences of the amino acid numbers 1-293 (V. Vincente et al., *J. Biol. Chem.*, 263, 18473-18479, 1988), and in the sequence of the amino acid numbers 251-285 (V. Vicente et al., *J. Biol. Chem.*, 265, 274-280, 1990), a partial protein

containing at least these regions may be sufficient.
[0029]

Further, the Fc region of immunoglobulin molecule may be derived from any animals, and may be of any subtype, and those that can be purified and/or detected with commercially available polyclonal antibodies and/or monoclonal antibodies, protein A, protein G or the like may be used. The immunoglobulin heavy chain comprises regions called VH domain, CH1 domain, hinge domain, CH2 domain and CH3 domain (and further CH4 domain in IgE) connected in this order from the amino terminus.

For example, the Fc region used for the chimeric protein may be a continuous sequence from the hinge domain to the CH3 domain of the above sequence. However, from the viewpoint that it should be able to be purified and/or detected with commercially available polyclonal antibodies and/or monoclonal antibodies, protein A, protein G or the like, the hinge domain is not essential, and it may partially contain a mutation such as deletion and insertion of one or more amino acid residues. Further, while the immunoglobulin may be derived from any animals including human and mouse, one derived from mouse can be used, for example. Although the subtype of the immunoglobulin may be any subtype, IgG can be used, for example. The subclass may also be any subclass, and IgG1, IgG2a and so forth can be mentioned, for example. Exemplary amino acid sequences of the chimeric protein of the present invention are shown in SEQ ID NOS: 7 and 14. In SEQ ID NOS: 7 and 14, it is presumed that 16 amino acid residues at the N-terminus constitute a signal peptide.

[0030]

The chimeric protein of the present invention

can be produced by allowing expression of a chimeric gene coding for it (chimeric protein gene) in a suitable cell. A chimeric protein gene can be prepared by obtaining a glycoprotein Iba chain gene and an immunoglobulin heavy chain gene respectively from a cDNA library, genomic library, DNA fragment or the like using genetic engineering techniques or chemically preparing them, and ligating them.

[0031]

A glycoprotein Iba chain gene can be obtained from, for example, a cDNA library produced by using a phage vector or the like from mRNA of HEL cell, which is a human megakaryocyte cell strain, through reverse transcription PCR using suitable primer DNA designed based on a known DNA sequence of glycoprotein Iba chain gene. Further, a clone containing a glycoprotein Iba chain gene can be obtained from such a cDNA library by performing hybridization using a probe DNA designed based on the known DNA sequence. Alternatively, it can be obtained by excising it from plasmid containing a glycoprotein Iba chain gene registered at ATCC (American Type Culture Collection, pGPIb2.4, deposition number: ATCC65755) with a suitable restriction enzyme.

[0032]

A gene of immunoglobulin heavy chain can be obtained from, for example, cDNA library produced from mRNA of mouse immunoglobulin producing hybridoma by using a phage vector or the like through reverse transcription PCR using suitable primer DNA designed based on a known DNA sequence of the immunoglobulin heavy chain gene. Further, a clone containing a mouse immunoglobulin gene can be obtained from such a cDNA library by performing hybridization using a probe DNA designed based on

the known DNA sequence.

[0033]

A chimeric protein gene can be obtained by digesting DNA strands of a full length glycoprotein Iba chain gene or a partial sequence thereof and a full length mouse immunoglobulin heavy chain $\gamma 1$ gene or $\gamma 2a$ gene or a partial sequence thereof with a suitable restriction enzyme and then ligating them. The digestion and the ligation of the both genes may be performed so that the ligation product should code for a chimeric protein consisting of an Fc region of immunoglobulin molecule fused at its amino terminus to a partial protein comprising a von Willebrand factor binding site of glycoprotein Iba chain at its carboxyl terminus. Further, extracellular secretion of the chimeric protein is desired, the segment for glycoprotein Iba chain may contain a signal peptide.

[0034]

A chimeric protein gene produced as described above is expressed by using a suitable host-vector system. As the host, animal cells, insect cells and so forth can be mentioned. The vector is not particularly limited so long as it can function as a vector in the host cell, and it is preferable to use an expression vector having a promoter suitable for the host cell. A chimeric protein can be produced by transforming the host cell with a recombinant vector obtained by inserting a chimeric protein gene into an expression vector, and culturing the transformed cell.

[0035]

While a chimeric protein produced as described above may be used as it is, it can be readily purified by utilizing the Fc region of immunoglobulin molecule through affinity

chromatography using immobilized protein A, protein G, anti-immunoglobulin antibodies and so forth.

[0036]

<2> Method for detecting binding of von Willebrand factor and glycoprotein Ib or inhibition of this binding

The first method for detecting the binding of von Willebrand factor and glycoprotein Ib or inhibition of the binding according to the present invention is characterized in that von Willebrand factor is immobilized in a reaction vessel in the presence of a substance inducing the binding of von Willebrand factor and glycoprotein Ib (henceforth also referred to as "binding inducing substance"), and the immobilized von Willebrand factor is allowed to react with the glycoprotein Ib.

[0037]

By immobilizing von Willebrand factor in a reaction vessel in the presence of a binding inducing substance, a step of adding a binding inducing substance during the reaction of von Willebrand factor and glycoprotein Ib in a liquid phase can be omitted.

[0038]

von Willebrand factor can be prepared from human blood according to the method described in H.R. Gralnick et al., *J. Clin. Invest.*, 62, 496 (1978) or the like.

As the binding inducing substance, there can be mentioned botrocetin, ristocetin and so forth, and botrocetin is preferred.

[0039]

As the reaction vessel in which von Willebrand factor is immobilized, a vessel made of synthetic resin such as polystyrene and polycarbonate or glass may be used. More specifically, a 96-well multi-

well plate made of polystyrene and so forth can be mentioned. By injecting a solution containing von Willebrand factor into the aforementioned reaction vessel, von Willebrand factor can be immobilized on a wall surface of the vessel. It is also possible to immobilize collagen on a wall surface of the reaction vessel, and allow von Willebrand factor to bind to the collagen. The conditions for immobilizing von Willebrand factor or collagen to a reaction vessel are not particularly limited so long as they can be immobilized. However, when a vessel made of polystyrene is used, for example, it is preferable to use a neutral solution, preferably at pH 6.8-7.8, more preferably at about pH 7.4.

[0040]

For the immobilization of von Willebrand factor, while a solution containing von Willebrand factor and a solution containing a binding inducing substance may be separately added to a reaction vessel, it is preferable to prepare a solution containing both of von Willebrand factor and a binding inducing substance, and add it into the reaction vessel, from the viewpoint of operation efficiency. Further, a reaction vessel in which von Willebrand factor is immobilized is preferably added with a bovine serum albumin solution or the like to block unbound areas on the wall surface.

[0041]

After von Willebrand factor is immobilized in a reaction vessel, the reaction vessel is washed and then glycoprotein Ib is added. Upon addition of glycoprotein Ib, the binding reaction of von Willebrand factor and glycoprotein Ib is caused. This reaction is attained in a liquid phase. Subsequently, the binding of von Willebrand factor and glycoprotein Ib is detected. This detection can

be performed by the method usually used for detection of the binding of von Willebrand factor and glycoprotein Ib.

[0042]

The second method according to the present invention is a method wherein the binding of von Willebrand factor and glycoprotein Ib or inhibition of the binding is detected by allowing the aforementioned chimeric protein or the chimeric protein labeled with a labeling substance to bind to von Willebrand factor immobilized in a reaction vessel, and detecting the Fc region of the immunoglobulin molecule or the labeling substance. More specifically, a solution containing von Willebrand factor is added to the reaction vessel to immobilize the von Willebrand factor on a wall surface of the reaction vessel. Then, a solution containing a chimeric protein is added to the reaction vessel to allow the chimeric protein to bind to the immobilized von Willebrand factor. This binding can be induced by the presence of a binding inducing substance in the reaction system of the von Willebrand factor and the chimeric protein. Specifically, von Willebrand factor is immobilized in the reaction vessel in the presence of a binding inducing substance in a manner similar to that of the aforementioned first method, or the binding inducing substance is added at the same time as, or at a time point around the addition of the solution containing the chimeric protein to the reaction vessel.

[0043]

The chimeric protein binds to the immobilized von Willebrand factor at the von Willebrand factor binding site of glycoprotein Ib contained in the molecule. The detection of the chimeric protein

bound to von Willebrand factor as described above can be performed by, for example, detecting the Fc region of the immunoglobulin molecule contained in the molecule. For the detection of the Fc region, a method usually used for immunoassay can be used.

Specifically, for example, a labeled substance that specifically binds to the Fc region such as protein A, protein G, and anti-immunoglobulin antibodies is added to the reaction vessel, and the label is detected. As the labeling substance, there can be mentioned enzymes such as alkaline phosphatase and peroxidase, biotin, avidin, fluorescent substances such as fluorescein, compounds containing a fluorescent rare earth element such as europium and lanthanoids and so forth. Biotin or avidin is detected by further binding to them another labeling substance bound to avidin or biotin. Enzymes can be detected by adding a suitable substrate to cause an enzymatic reaction and observing visible absorbance, UV absorbance, fluorescence, luminescence etc. Furthermore, fluorescent substances and compounds having a property of emitting fluorescence can be detected based on fluorescence emitted upon irradiation with excitation light.

[0044]

The chimeric protein bound to the immobilized von Willebrand factor can also be detected by using a chimeric protein labeled with a labeling substance beforehand and detecting this labeling substance. The labeling substance and the detecting method therefor may be similar to those mentioned above for use in the detection of the Fc region. When a chimeric protein labeled with a labeling substance is used, a purified chimeric protein is preferably used.

The purification of the chimeric protein can be attained by using the Fc region of the immunoglobulin molecule through affinity chromatography and so forth as described above.

[0045]

In the aforementioned first and second methods, inhibition of the binding of von Willebrand factor and glycoprotein Ib can be detected by comparing a case where a substance inhibiting the binding of von Willebrand factor and glycoprotein Ib (henceforth also referred to as "binding inhibition substance") is added to a reaction vessel at substantially the same time as the addition of glycoprotein Ib (or a chimeric protein) to the reaction vessel or prior to the addition of glycoprotein Ib and a case where the inhibition substance is not added for the binding of von Willebrand factor and glycoprotein Ib.

[0046]

Further, in the aforementioned methods, a substance inhibiting the binding of von Willebrand factor and glycoprotein Ib can be detected by adding a sample containing a substance to be detected to the reaction vessel during the reaction of von Willebrand factor and glycoprotein Ib or prior to the reaction and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib. If a standard curve that represents the relation between amount of an inhibition substance and binding of von Willebrand factor and glycoprotein Ib is prepared, the inhibition substance of an unknown amount can be quantified.

[0047]

No low molecular weight compound has been reported so far, which inhibits the binding of von Willebrand factor and glycoprotein Ib and has antithrombotic activity. The methods of the present

invention are extremely simpler compared with the conventional methods, and are also useful for search of such a low molecular compound as mentioned above.

[0048]

<3> Method and kit for measurement of glyocalicin

In the aforementioned first and second methods, glyocalicin can be measured by adding a sample containing glyocalicin to the reaction vessel during the reaction of von Willebrand factor and glycoprotein Ib or prior to the reaction and detecting inhibition of the binding of von Willebrand factor and glycoprotein Ib. If a standard curve that represents the relation between glyocalicin concentration and the binding of von Willebrand factor and glycoprotein Ib is prepared, concentration of glyocalicin in an unknown amount can be measured.

[0049]

If von Willebrand factor and the chimeric protein are prepared as a kit, the measurement of glyocalicin according to the present invention can conveniently be performed. As such a kit, there can be specifically exemplified a kit comprising von Willebrand factor, a chimeric protein, a binding inducing substance, glyocalicin of a known amount, anti-immunoglobulin antibodies labeled with alkaline phosphatase or the like, a reagent for detecting the label and a washing buffer. As another embodiment, there can be exemplified a kit comprising von Willebrand factor, a chimeric protein labeled with a labeling substance, a binding inducing substance, glyocalicin of a known amount, a reagent for detecting the label and a washing buffer.

[0050]

[Examples]

The present invention will be explained more

specifically in to the following examples.

[0051]

[Example 1] Preparation of chimeric protein gene

<1> Cloning of glycoprotein Iba chain gene

Cloning of human glycoprotein Iba chain gene was attained by constructing a cDNA library from human erythroleukemia cells (HEL) according to the method described in Molecular Cloning (Sambrook, J., Fritsch, E.F., Maniatis, T., Molecular Cloning, Cold Spring Harbor Laboratory Press (1989)). That is, human erythroleukemia cells were stimulated by culturing them in a medium containing 160 nM of a phorbol ester (phorbol-12-myristate-13-acetate: PMA) for 48 hours, and then the medium was removed. A guanidinium thiocyanate buffer (4.0 M guanidinium thiocyanate, 0.1 M Tris-HCl (pH 7.5), 1% 2-mercaptoethanol) was added to the cells to suspend the cells in the buffer. The cell suspension was subjected to disruption treatment by using a Polytron homogenizer (produced by Brinkmann).

[0052]

Laurylsarcosinate (sodium laurylsarcosinate) was added to the disrupted cell suspension at a final concentration of 0.5%. This solution was centrifuged at 5000 x g for 10 minutes to remove the precipitates. The centrifugation supernatant was overlaid on cesium chloride/EDTA solution (5.7 M CsCl, 0.01 M EDTA, pH 7.5) contained in an ultracentrifugation tube and subjected to ultracentrifugation at 100000 x g for 20 hours. The precipitated RNA was collected and purified by ethanol precipitation to obtain total RNA.

[0053]

The obtained total RNA was loaded on an oligo-dT cellulose column to obtain mRNA. From 10 µg of this mRNA, single-stranded DNA was prepared by using

random hexamer oligo DNA as a primer and a reverse transcriptase, and then double-stranded cDNA was prepared by using a DNA polymerase. An *EcoRI* adapter was ligated to this cDNA by using T4 DNA ligase. The cDNA to which the adapter was ligated was subjected to a phosphorylation treatment using T4 polynucleotide kinase, and purified by using a gel filtration column. A λ gt10 arm prepared so that it could be inserted into an *EcoRI* restriction site (produced by Stratagene) was ligated to this DNA using T4 DNA ligase. This recombinant DNA was packaged in phage to obtain a cDNA library.

[0054]

Escherichia coli NM514 was infected with this phage. Plaque hybridization was performed for the produced phage plaques by using oligo DNA (SEQ ID NO: 1) end-labeled with a radioisotope (^{32}P) as a probe. That is, the produced phage plaques were transferred to a nitrocellulose filter, and DNA was denatured with an alkaline denaturation solution (0.5 M sodium hydroxide, 1.5 M sodium chloride). The filter was neutralized with a neutralization solution (0.5 M Tris-HCl, pH 7.0, 1.5 M sodium chloride), and heated at 80°C for 2 hours to immobilize the DNA on the filter. Synthesized DNA (chemically synthesized by using a DNA synthesizer Model 380A produced by Perkin-Elmer Applied Biosystems) was labeled at the 5' end of the DNA with γ - ^{32}P -ATP with the aid of T4 DNA kinase (produced by Takara Shuzo), and used as probe DNA. The nucleotide sequence of the aforementioned oligo DNA was designed based on the nucleotide sequence of a known human glycoprotein Iba chain gene (J.A. Lopez et al., *Proc. Natl. Acad. Sci. USA*, 84, 5615-5619 (1987)).

[0055]

The nitrocellulose filter (diameter: 132 mm) on which phage plaque DNA was transferred was immersed in 4 ml of a hybridization buffer (0.9 M sodium chloride, 0.09 M sodium citrate (pH 7.0), 0.5% sodium laurylsulfate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 100 µg/ml heat-denatured salmon sperm DNA) containing the probe corresponding to 1×10^6 cpm (count per minute) per one filter, and allowed to hybridize at 42°C for 16 hours. The filter was washed three times with 1 x SSC (0.875% sodium chloride, 0.441% sodium citrate, pH 7.0) and 0.1% sodium laurylsulfate solution at 37°C for 30 minutes to remove the probe non-specifically adsorbed on the filter. After the filter was dried, radioautography was performed by using an X-ray film. As a result, four strains of positive clones were obtained.

[0056]

The phage was isolated from each positive clone, and *Escherichia coli* NM514 was infected with the isolated phage and proliferated. Then, phage DNA from each clone was purified by cesium chloride density gradient ultracentrifugation. This phage DNA was digested with a restriction enzyme *EcoRI*, and DNA was purified by agarose electrophoresis. This purified DNA was inserted into the *EcoRI* site of pBluescriptSK- (produced by Stratagene) and used for transformation of the *Escherichia coli* XLIIblue (produced by Stratagene) to obtain a transformant. Plasmid was prepared from the transformant by the alkali SDS method, and the nucleotide sequence of the plasmid DNA was determined by the dideoxy method using a DNA sequencer Model 377 produced by Perkin-Elmer Applied Biosystems according to the protocol attached to the instrument. It was confirmed that one strain among the obtained positive clones

contained cDNA of 2.4 kb, and it was the clone having the full length of human glycoprotein Iba gene reported by J.A. Lopez et al. (*Proc. Natl. Acad. Sci. USA*, Vol. 84, pp.5615-5619 (1987)). This plasmid was designated as pBluescriptGPIbAlpha.

[0057]

<2> Cloning of gene coding for Fc region of immunoglobulin γ 1 origin)

The gene for the Fc region of mouse immunoglobulin γ 1 was obtained by extracting total RNA from a mouse hybridoma cell strain MB40.3 and performing reverse transcription PCR. That is, from 10 ml of culture broth of MB40.3 cells, the cells were collected by centrifugation, and the cells were lysed with ISOGEN (1 ml, produced by Nippon Gene). The lysate was subjected to syringing using an injection needle of 18G. The lysate was left for 5 minutes, then added with 200 μ l of chloroform and mixed. The mixture was left stand for 2 minutes and then centrifuged (15000 rpm, 15 minutes) to recover an aqueous phase. The aqueous phase was added with 500 μ l of 2-propanol, mixed, left stand for 5 minutes and centrifuged (15000 rpm, 15 minutes) to precipitate the total RNA. The total RNA was washed with 75% ethanol and dissolved in 100 μ l of sterilized water.

[0058]

cDNA was prepared by using 3 μ g (20 μ l) of MB40.3 cell total RNA prepared as described above as a template and using random primers and reverse transcriptase (Superscript II produced by GIBCO). The cDNA was amplified by PCR using the primers of SEQ ID NOS: 2 and 3, digested with HindIII and BamHI, purified by agarose gel electrophoresis, and ligated to pGEM-3Zf (produced by Promega) digested with HindIII and BamHI. *Escherichia coli* XLIIblue

(produced by Stratagene) was transformed with the obtained recombinant DNA. One of the obtained transformants was cultured. Plasmid was prepared by the alkali SDS method, and the nucleotide sequence thereof was determined by the dideoxy method using a DNA sequencer Model 377 produced by Perkin-Elmer Applied Biosystems according to the protocol attached to the instrument. The obtained nucleotide sequence of the gene fragment for the Fc region of mouse immunoglobulin $\gamma 1$ is shown in SEQ ID NO: 4. This plasmid was designated as pGEMmIgG1Fc.

[0059]

<3> Preparation of plasmid expressing chimeric protein (GP1b-mIgG1Fc)

A chimeric protein comprising the human glycoprotein Ib gene and the Fc region of mouse immunoglobulin $\gamma 1$ obtained as described above, which were fused together, was prepared as follows.

First, the plasmid pBluescriptGP1Alpha containing the glycoprotein Ib α chain gene was digested with restriction enzymes *EcoRI* and *XbaI*, and separated by agarose gel electrophoresis to recover DNA of about 1000 bp, which corresponded to the N-terminus region of glycoprotein Ib α chain gene. This was inserted into the *EcoRI*-*XbaI* site of pBluescriptSK- (produced by Stratagene) to prepare plasmid pBluescriptGP1bEX.

[0060]

Separately, the plasmid pGEMmIgG1Fc containing the partial gene of mouse immunoglobulin $\gamma 1$ obtained as described above was digested with a restriction enzyme *XbaI* and separated by agarose gel electrophoresis to recover the IgG1Fc gene of 700 bp. This DNA was ligated to pBluescriptGP1bEX digested with a restriction enzyme *XbaI* and subjected to CIAP treatment to obtain plasmid pBluescriptGP1bIgG1FcFH.

The protein encoded by this gene was designated as GPIb-mIgG1Fc, of which gene sequence and amino acid sequence are shown in SEQ ID NOS: 6 and 7, respectively. In SEQ ID NO: 6, it is presumed that the 16 amino acid residues of the N-terminus constitute a signal peptide.

[0061]

Further, pBluescriptGPIbIgG1FcFH was digested with a restriction enzyme XhoI, and DNA coding for GPIbFcFH was separated by agarose gel electrophoresis. This DNA was inserted into the XhoI site of an expression vector for animal cells pSD(X) to obtain an expression vector pSDGPIbIgG1FcFH, in which the GPIb gene was inserted downstream from a promoter. The outline of the aforementioned procedure is shown in Fig. 1.

Escherichia coli XLIIblue (*Escherichia coli* AJ13434) harboring the plasmid pSDGPIbIgG1FcFH was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on April 2, 1998, and given an accession number of FERM P-16749.

[0062]

<4> Cloning of gene coding for Fc region of immunoglobulin (γ 2a origin)

The gene of the Fc region of mouse immunoglobulin γ 2a was obtained by extracting total RNA from a mouse hybridoma cell strain W6/32 and performing reverse transcription PCR. That is, from 10 ml of culture broth of W6/32 cells, the cells were collected by centrifugation, and the cells were lysed with ISOGEN (1 ml, produced by Nippon Gene). The lysate was subjected to syringing using an injection needle of 18G. The lysate was left for 5 minutes, then added with 200 μ l of chloroform and

mixed. The mixture was left stand for 2 minutes and then centrifuged (15000 rpm, 15 minutes) to recover an aqueous phase. The aqueous phase was added with 500 µl of 2-propanol, mixed, left stand for 5 minutes and then centrifuged (15000 rpm, 15 minutes) to precipitate the total RNA. The total RNA was washed with 75% ethanol and dissolved in 100 µl of sterilized water.

[0063]

cDNA was prepared by using 3 µg (20 µl) of the W6/32 cell total RNA prepared as described above as a template and using random primers and reverse transcriptase (Superscript II produced by GIBCO).. The cDNA was amplified by PCR using primers having nucleotide sequences of SEQ ID NOS: 8 and 9, digested with *Hind*III and *Bam*HI, purified by agarose gel electrophoresis, and ligated to pGEM-3Zf (produced by Promega) digested with *Hind*III and *Bam*HI. *Escherichia coli* XLIIblue (produced by Stratagene) was transformed with the obtained recombinant DNA. One of the obtained transformants was cultured. Plasmid was prepared by the alkali SDS method and the nucleotide sequence thereof was determined by the dideoxy method using a DNA sequencer Model 377 produced by Perkin-Elmer Applied Biosystems according to the protocol attached to the instrument. The obtained nucleotide sequence of the gene fragment for the Fc region of mouse immunoglobulin γ2a is shown in SEQ ID NO: 10. This plasmid was designated as pGEMmIgG2aFc.

[0064]

<5> Preparation of plasmid expressing chimeric protein (GP1b-mIgG2aFc)

A chimeric protein gene comprising the human glycoprotein Ib gene and the Fc region of mouse immunoglobulin γ1 obtained as described above and

fused together was prepared as follows.

[0065]

First, the plasmid pBluescriptGPIAlpha containing the glycoprotein Ib α chain gene was digested with restriction enzymes *EcoRI* and *XbaI*, and separated by agarose gel electrophoresis to obtain a *KpnI*-*XbaI* DNA fragment containing the sequence of glycoprotein Ib gene for the sequence of from the N-terminus to the 319th aspartic acid.

[0066]

Further a gene fragment of the Fc region of mouse immunoglobulin γ 2a having an *XhoI* site at the 5' end side and an *XbaI* site at the 3' end side was produced by PCR (annealing temperature: 55°C, 30 cycles) using the plasmid pGEMmIgG2aFc containing the partial gene of mouse immunoglobulin γ 2a obtained as described above, two kinds of synthetic primers having the nucleotide sequences shown in SEQ ID NOS: 9 and 12 and PFU (produced by Stratagene). This gene fragment was digested with *XbaI* and *XhoI*, then purified by agarose gel electrophoresis and ligated to pBluescriptSK- digested with *XbaI* and *XhoI*. *Escherichia coli* XLIIblue (produced by Stratagene) was transformed with the obtained recombinant plasmid. Plasmid was prepared from the obtained transformant by the alkali SDS method, and the nucleotide sequence thereof was determined by the dideoxy method using a DNA sequencer Model 377 produced by Perkin-Elmer Applied Biosystems according to the protocol attached to the instrument. As a result, it was confirmed to have a nucleotide sequence corresponding to the nucleotide sequence shown in SEQ ID NO: 10 of which 6 nucleotides at the 5' end was replaced with TCTAGAC and 6 nucleotides at the 3' end was eliminated. This plasmid was designated as pBluescriptmIgG2a. This plasmid was

digested with *Xba*I and *Xho*I, and purified by agarose gel electrophoresis to obtain a *Xba*I-*Xho*I fragment of the Fc region gene of mouse immunoglobulin γ 2a. [0067]

The *Kpn*I-*Xba*I fragment of human glycoprotein Ib gene and the *Xba*I-*Xho*I fragment of Fc region gene of mouse immunoglobulin γ 2a obtained as described above were ligated to pBluescriptSK- digested with *Kpn*I and *Xho*I, and *Escherichia coli* XLIIblue (produced by Stratagene) was transformed with the obtained recombinant plasmid. One of the obtained transformants was cultured, and plasmid was prepared by the alkali SDS method to obtain plasmid containing a gene coding for a protein (chimeric protein) comprising the N-terminus side region of glycoprotein Ib (amino acid numbers 1-319, including a signal peptide) and the Fc region of mouse immunoglobulin γ 2a bound together (SEQ ID NO: 13). This plasmid was designated as pBluescriptGPIbFc2a, and the encoded chimeric protein corresponding to the gene was especially designated as GPIb-mIgG2aFc, of which amino acid sequence was shown in SEQ ID NO: 14. In SEQ ID NO: 14, it is presumed that 16 amino acid residues of the N-terminus constitute a signal peptide. *Escherichia coli* XLIIblue (*Escherichia coli* AJ13432) harboring the plasmid pBluescriptGPIbFc2a was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on March 19, 1998, and given an accession number of FERM P-16719. [0068]

pBluescriptGPIbFc2a was digested with *Xho*I, and purified by agarose electrophoresis. This *Xho*I fragment containing a gene of the chimeric protein was ligated to the *Xho*I site of the same animal cell

expression vector pSD(x) as the above <3> to obtain plasmid pSDGPIbFc2a. Further, pGPIbFcbluescript was digested with *EcoRI* and *XhoI*, and the *EcoRI*-*XhoI* fragment containing the chimeric protein gene was inserted into the *EcoRI*-*XhoI* site of expression vector pMikNeo(+) for animal cells (kindly provided by Dr. K. Maruyama, the Institute of Medical Science, the University of Tokyo) having SR α promoter (K. Maruyama and Y. Takebe *et al.*, *Medical Immunology*, 20, 27-32, 1990) to obtain plasmid pMikGPIbFc. The outline of the procedure used for obtaining pMikGPIbFc is shown in Fig. 2.

[0069]

[Example 2] Production of chimeric protein (GPIb-mIgG1Fc) using animal cells

Cells producing the chimeric protein were produced as follows. CHODhfr⁻ cells were cultured by using D-MEM medium (10 ml, produced by GIBCO) containing 10% fetal bovine serum at 37°C under 5% CO₂ at a density of 5 x 10⁵ cells per 10-cm dish. The cells were transfected with pSDGPIbIgG1Fc prepared as described in Example 1 <3>. The transfection was performed by using calcium phosphate as described below. That is, about 10 μ g per 10-cm dish of pSDGPIbIgG1Fc was added to 0.5 ml of BES buffer (pH 6.96) containing 0.125 M calcium chloride, uniformly added dropwise to a dish, and incubated overnight at 35°C under 3% CO₂. Then, the dish was washed twice with PBS, and further incubated in α -MEM medium not containing nucleic acid at 37°C for about 24 hours under 5% CO₂. The cells transfected as described above were further cultured in α -MEM medium not containing nucleic acids, but containing 0.05 μ M methotrexate (MTX) and 10% fetal bovine serum to obtain chimeric protein producing cells.

[0070]

The chimeric protein producing cells obtained as described above were cultured in an F175 cell culture flask containing α -MEM medium not containing nucleic acid, but containing 0.05 μ M methotrexate (MTX) and 10% fetal bovine serum, until about 60% confluent. Then, the medium was exchanged with a serum-free medium, ASF104 medium (produced by Ajinomoto), containing 0.05 μ M methotrexate (MTX), and the culture supernatant was collected four days later.

[0071]

[Example 3] Production of chimeric protein (GP1b-mIgG2aFc) using animal cells

Cells producing the chimeric protein were produced as follows. CHOK1 cells were cultured by using D-MEM medium (10 ml, produced by GIBCO) containing 10% fetal bovine serum at 37°C under 5% CO₂ at a density of 5×10^5 cells per 10-cm dish. The cells were transfected with pMikGP1bFc prepared in Example 1. The transfection was performed by the calcium phosphate method as described below. That is, about 10 μ g per 10-cm dish of pMikGP1bFc was added to 0.5 ml of BES buffer (pH 6.96) containing 0.125 M calcium chloride, uniformly added dropwise to a dish, and incubated overnight at 35°C under 3% CO₂. Then, the dish was washed twice with PBS, and further incubated in D-MEM medium at 37°C for about 24 hours under 5% CO₂. The cells transfected as described above were further cultured in D-MEM medium containing G418 (850 μ g/ml) and 10% fetal bovine serum to obtain chimeric protein producing cells, which were G418 resistant cells.

[0072]

The chimeric protein producing cells obtained as described above were cultured in an F175 cell

culture flask containing D-MEM medium containing G418 (800 µg/ml) and 10% fetal bovine serum until about 60% confluent. Then, the medium was exchanged with a serum-free medium, ASF104 medium (produced by Ajinomoto), containing G418 (800 µg/ml), and the culture supernatant was collected four days later. [0073]

The collected culture supernatant was centrifuged to remove the solid, and then 160 ml of the supernatant was passed through a Protein A Hitrap (1 ml, produced by Pharmacia) column washed with 20 mM phosphate buffer (pH 7.0) so that the chimeric protein should be adsorbed on the column. The column was sufficiently washed with 20 mM phosphate buffer (pH 7.0), and then eluted with 0.1 M citrate buffer (pH 4.5). The elution of the chimeric protein was performed with detection by a UV monitor at 280 nm, and chimeric protein eluted fractions were immediately neutralized by adding 1 M Tris-HCl buffer (pH 8.5). As a result of SDS electrophoresis, the chimeric protein obtained as described above was found to be a protein having a molecular weight of about 80 kDa as a reduced form and a molecular weight about twice as much as that of the reduced form as non-reduced form. [0074]

[Example 4] Detection of binding of chimeric protein to immobilized mixture of von Willebrand factor and botrocetin

<1> Detection of binding of chimeric protein by ELISA using anti-mouse IgG-Fc antibodies

Botrocetin was obtained from 1 g of lyophilized product of crude venom of *Botrops jararaca* (produced by Sigma) by purification according to the method reported by Read (M.S. Read et al., *Proc. Natl. Acad. Sci. USA.*, 75, 4514-4518, 1978).

[0075]

Immobilization of a mixed solution of von Willebrand factor and botrocetin on a 96-well multititer plate was attained as follows. First, a physiological saline solution of von Willebrand factor (250 µg/ml) and a physiological saline solution of botrocetin (500 µg/ml), which were prepared in a conventional manner, were appropriately diluted, and mixed at the concentration ratios shown in Fig. 2. Then, 50 µl of each mixture was added to each well of a 96-well multititer plate (Maxisorp, produced by Nunc). The plate was left stand overnight at 4°C, and then each well was washed once with a physiological Tris buffer (150 µl, 20 mM Tris-HCl (pH 7.4), 0.15 M sodium chloride; Tris buffered saline, referred to as "TBS" hereinafter). Then, each well was added with 100 µl of TBS containing 10% BSA (bovine serum albumin), left stand for about 3 hours, and washed 3 times with TBS to obtain a von Willebrand factor immobilized plate.

[0076]

Each well of the plate on which von Willebrand factor was immobilized in the presence of botrocetin as described above was added with 25 µl of TBS containing 1% BSA and 25 µl of a solution prepared by diluting 8 times the culture supernatant of the chimeric protein (GP1b-mIgG1Fc) producing cells obtained by using the serum free medium with TBS containing 1% BSA, incubated at room temperature for 1 hour, and washed 3 times with TBS (150 µl) containing 0.05% Tween-20. Anti-mouse IgG-Fc goat polyclonal antibodies (Catalog No. 55482, produced by Organon Teknika) were biotinylated by using Biotin Labeling Kit (Catalog No. 1418165, produced by Boehringer Mannheim) according to the protocol

attached to the kit. 50 μ l of 0.1% BSA/TBA solution containing about 2 μ g/ml of the above biotinylated anti-mouse IgGFc antibodies was added to each well of the plate, and incubated at room temperature for 1 hour. Further, each well was washed 3 times with TBS (150 μ l) containing 0.05% Tween-20, added with 50 μ l of a solution of the reagent (mixture of biotinylated alkaline phosphatase and streptavidin) contained in VECTASTAIN ABC kit (kit for biotin detection, Alkaline phosphatase standard, Catalog No. AK-5000, produced by Vector Laboratories), which solution was prepared in 0.1% BSA/TBS at 1/5 concentration of that used in the method specified in the manual, and incubated at room temperature for 1 hour. Each well was washed 5 times with TBS (150 μ l) containing 0.05% Tween-20, and added with 100 μ l of 100 mM NaHCO₃ solution containing 10 mM MgCl₂, in which p-nitrophenylphosphate was dissolved at 1 mg/ml, to carry out the color development reaction for about 1 hour. After the color development, absorbance at 405 nm was measured. As shown in Fig. 3, the binding of the chimeric protein was observed in a botrocetin and von Willebrand factor amount dependent manner.

[0077]

<2> Detection of chimeric protein binding using europium (Eu) labeling method

The chimeric protein (GPIb-mIgG2aFc) solution purified by the Protein A column, which was obtained in Example 3, was dialyzed against physiological saline. The solution of about 200 μ g/1.5 ml was concentrated to 780 μ l (concentration of about 250 μ g/ml) by ultrafiltration using Centricon-10 (produced by Amicon). 500 μ l of the concentrated solution (containing about 125 μ g of GPIb-mIgG2aFc) was added with 50 μ l of 0.5 M NaHCO₃, then added

with 50 μ l of a solution obtained by dissolving 0.2 mg of Eu-Labeling Reagent (europium DTTA-isothiocyanate as compound, DELFIA 1244-302, produced by Wallac) in 250 μ l of physiological saline, and stirred at room temperature for about 40 hours to allow the reaction of europium DTTA-isothiocyanate.

[0078]

The above reaction mixture was subjected to gel filtration using HiLoad16/60 Superdex 75pg (inner diameter of 16 mm, length of 60 cm, produced by Pharmacia) to separate the unreacted reagent and the chimeric protein. The gel filtration was performed at a flow rate of 1 ml/minute by using physiological saline as the eluant. The chimeric protein labeled with Eu was recovered in fractions of the elution volume of 40 to 48 ml. The protein was quantified by using a protein assay kit (Protein Assay, produced by Bio-Rad) and IgG as a standard substance. As a result, the concentration of the labeled chimeric protein in the eluted solution had a concentration of 6.4 μ g/ml. Hereafter, the following experiments were conducted by using this value as the chimeric protein concentration.

[0079]

The binding of the europium (Eu) labeled chimeric protein and the von Willebrand factor immobilized in the presence of botrocetin prepared as described above was detected as follows. According to the method mentioned in Example 4 <1>, a mixed solution (TBS) containing 2.5 μ g/ml of von Willebrand factor and 2.5 μ g/ml of botrocetin was added to each well of a 96-well multititer plate (microtitration plate DELFIA, 1244-550, produced by Pharmacia Biotech), immobilized overnight and subjected to washing, blocking and washing to

prepare a von Willebrand factor immobilized plate.
[0080]

Each well of the above plate was added with 25 μ l of an assay buffer containing 0.5% BSA (Assay Buffer, Wallac DELFIA 1244-106, produced by Pharmacia Biotech, Composition: 0.5% BSA, 0.05% bovine γ -globulin, 0.01% Tween-40, 20 μ M DTPA (diethylenetriamine tetraacetic acid), 50 mM Tris-HCl buffered saline (pH 7.8), 0.05% sodium azide) or the recombinant AS1051 (in which Cys81 was replaced with Ala, N. Fukuchi et al., WO 95/08573) at a final concentration of 20 μ g/ml as the binding inhibition substance, further added with 25 μ l of a solution of europium (Eu) labeled chimeric protein in the same assay buffer (100 ng/ml), shaken for 1 minute for stirring, and then left stand at room temperature for 2 hours. Each well of the plate was washed 5 times with TBS (150 μ l) containing 0.05% Tween-20, then added with 100 μ l of a fluorescence enhancement buffer (Enhancement buffer, 1244-104, produced by Pharmacia Biotech, Composition: 15 μ M β -NTA (2-naphthoyltrifluoroacetone), 50 μ M TOPO (tri-n-octylphosphine oxide), 1 g/L Triton X-100, 100 mM acetic acid/potassium hydrogen phthalate buffer), and shaken for 1 minute for stirring. Then, the amount of europium (Eu) was measured by using a DELFIA Research fluorophotometer (1230 ARCUS Fluorometer, produced by LKB). The measured values (with addition or no addition of the binding inhibition substance) and CV value (deviation, %) are shown in Table 1.

[0081]

[Table 1] Measured value and CV value (%) by Eu-labeling method

Average value of count in control (n=80)	26668 cpm
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CV value (%)	6.75%
Average value of count with addition of AS1051 (10 µg/ml) (n=6)	935 cpm
S/N ratio	28.5

[0082]

[Example 5] Detection of inhibition by binding inhibition substance for binding of von Willebrand factor and chimeric protein

<1> Detection of inhibition for binding of chimeric protein by ELISA using anti-mouse IgG-Fc antibodies

The detection was carried out in the same manner as that of Example 4 <1> except that von Willebrand factor was immobilized by using a mixed solution (TBS) containing 2.5 µg/ml of von Willebrand factor and 2.5 µg/ml of botrocetin, and a binding inhibition substance of which inhibitory activity was desired to be measured was added to the reaction of the immobilized von Willebrand factor and the culture supernatant of the chimeric protein producing cells.

[0083]

As the binding inhibition substance, AJvW-2, which is an anti-human von Willebrand factor monoclonal antibody, and a human glycoprotein Ib binding peptide derived from snake venom of *Crotalus horridus horridus* were used.

[0084]

The hybridoma producing AJvW-2 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on August 24, 1994, and given an accession number of FERM P-14487. Then, it was transferred to an international deposit under the provisions of the Budapest Treaty on September

29, 1995, and given an accession number of FERM BP-5248 (refer to WO96/17078). AJvW-2 can be obtained by culturing this hybridoma.

[0085]

The aforementioned human glycoprotein Ib binding peptide corresponded to a single chain peptide obtained from a multi-mer peptide derived from snake venom of *Crotalus horridus horridus* (AS1051) in which 81-cysteine residue was replaced with an alanine residue (variant type AS1051). The variant type AS1051 was obtained by modifying the gene coding for AS1051 so that the 81-cysteine residue should be replaced with an alanine residue, and expressing it in *Escherichia coli*. *E. coli* HB101/pCHA1 (*E. coli* AJ13023) harboring pCHA1 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on August 12, 1994 as an international deposit under the provisions of the Budapest Treaty, and given an accession number of FERM BP-4781 (refer to WO95/08573). AS1051 itself is also a human glycoprotein Ib binding peptide, and it can be detected in the same manner as that for the variant type AS1051.

[0086]

The inhibitory activities of AJvW-2 and the variant type AS1051 for the binding of the chimeric protein (i.e., glycoprotein Ib) are shown in Fig. 4.

[0087]

<2> Detection of inhibition for binding of chimeric protein by europium (Eu) labeling method

The detection was carried out in the same manner as that of Example 4 <2> except that von Willebrand factor was immobilized by using a mixed

solution (TBS) containing 2.5 µg/ml of von Willebrand factor and 2.5 µg/ml of botrocetin, and a binding inhibition substance of which inhibitory activity was desired to be measured was added to the reaction of the immobilized von Willebrand factor and the chimeric protein labeled with europium (Eu).
[0088]

As the binding inhibition substance, AJvW-2, which is an anti-human von Willebrand factor monoclonal antibody, and the variant type AS1051. The inhibitory activities of the both substances for the binding of the chimeric protein (i.e., glycoprotein Ib) are shown in Fig. 5.

[0089]

[Example 6] Detection of glyocalicin in plasma
<1> Detection of glyocalicin by ELISA using anti-mouse IgG-Fc antibodies

Human plasma was prepared by collecting blood from healthy volunteers using an injection needle of 18G, adding 1/10 volume of 3.8% aqueous sodium citrate solution to the blood, and centrifuging the mixture at 3000 x rpm for 10 minutes to separate a supernatant.

[0090]

Each human plasma collected independently from three volunteers was successively diluted 2-fold (8 times of dilution in total), and 25 µl of the plasma was added to each well of the plate. Each well of the plate was further added with 25 µl of a solution prepared by diluting 8-fold a culture supernatant obtained from culture of the chimeric protein producing cells in a serum-free medium with TBS containing 0.1% BSA, and incubated at room temperature for 1 hour. The subsequent reactions and color development were performed in the same manner as Example 5 <1>, and the average values of

the results are shown in Fig. 6.

[0091]

The blood concentration of glyocalicin in healthy people was reported to be about 2 µg/ml. On the other hand, the glyocalicin concentration showing 50% binding inhibition in this detection system was about 400 ng/ml. From this fact, it was considered that a glyocalicin amount of 60 ng/ml or more could sufficiently be measured in view of the linearity of the plot.

[0092]

<2> Detection of glyocalicin using chimeric protein labeled with europium (Eu)

Each human plasma independently prepared in the same manner as the above <1> were successively diluted 2-fold with TBS (8 times of dilution in total), and 25 µl each of the diluted plasma was added to each well of a von Willebrand factor immobilized plate prepared in the same manner as in Example 5 <1> (microtitration plate DELFIA, 1244-550, produced by Pharmacia Biotech, was used as the base plate). Further, 25 µl a solution of the chimeric protein labeled with europium (Eu) in assay buffer prepared in the same manner as in Example 4 <1> (100 ng/ml, Assay Buffer; 1244-106, produced by Pharmacia Biotech) was added to each well of the plate for reaction. The subsequent washing and measurement were performed in the same manner as in Example 5 <2>, and the average values of the results are shown in Fig. 7.

[0093]

The blood concentration of glyocalicin in healthy people was reported to be about 2 µg/ml. On the other hand, the glyocalicin concentration showing 50% binding inhibition was about 60 ng/ml in this detection system. From this fact, it was

considered that a glyocalicin amount of 30 ng/ml or more could sufficiently be measured.

[0094]

[Advantageous effect of the invention]

According to the present invention, the binding of glycoprotein Ib and von Willebrand factor or inhibition thereof can be detected in a simple manner. According to the method of the present invention, there are provided a simple method for quantification of glyocalicin with superior quantification ability, and a simple method for measurement of a substance inhibiting the binding of von Willebrand factor and glycoprotein Ib with superior operability.

[0095]

If von Willebrand factor is immobilized in the presence of a binding inducing substance such as botrocetin, the binding of von Willebrand factor and glycoprotein Ib can be observed in a simple manner with good reproducibility, without adding a binding inducing substance such as botrocetin or ristocetin to a liquid phase.

[0096]

Further, by utilizing the chimeric protein of the present invention, it becomes unnecessary to prepare or obtain monoclonal antibodies for detection or quantification of a binding inhibition substance such as glyocalicin.

Moreover, the present invention also provides a method for preparing a chimeric molecule (chimeric protein) that comprises a partial protein of glycoprotein Ib bound to the Fc region of immunoglobulin molecule by using animal cells.

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[0097]

[Sequence Listing]

SEQUENCE ID NO: 1

Length: 36

Type: nucleic acid

Number of chain: 1

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

atctgtgagg tctccaaagt ggccagccac ctagaa

36

[0098]

SEQUENCE ID NO: 2

Length: 29

Type: nucleic acid

Number of chain: 1

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

atatctagat gtgccaggg attgtggtt

29

[0099]

SEQUENCE ID NO: 3

Length: 36

Type: nucleic acid

Number of chain: 1

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

ataaagcttc tctgagtcatt taccaggaga gtggga

36

[0100]

SEQUENCE ID NO: 4

Length: 684

Type: nucleic acid

Number of chain: 2

Topology: linear

Type of sequence: cDNA to mRNA

Sequence:

```

gtg ccc agg gat tgt ggt tgt aag cct tgc ata tgt aca gtc cca gaa 48
Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu
  1           5           10           15
gta tca tct gtc ttc atc ttc ccc cca aag ccc aag gat gtg ctc acc 96
Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr
      20           25           30
att act ctg act cct aag gtc acg tgt gtt gtg gta gac atc agc aag 144
Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Lys
      35           40           45
gat gat ccc gag gtc cag ttc agc tgg ttt gta gat gat gtg gag gtg 192
Asp Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val
      50           55           60
cac aca gct cag acg caa ccc cgg gag gag cag ttc aac agc act ttc 240
His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe
      65           70           75           80
cgc tca gtc agt gaa ctt ccc atc atg cac cag gac tgg ctc aat ggc 288
Arg Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly
      85           90           95
aag gag ttc aaa tgc agg gta aac agt gca gct ttc cct gcc ccc atc 336
Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile
      100          105          110
gag aaa acc atc tcc aaa acc aaa ggc aga ccg aag gct cca cag gtg 384
Glu Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val
      115          120          125
tac acc att cca cct ccc aag gag cag atg gcc aag gat aaa gtc agt 432
Tyr Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser
      130          135          140
ctg acc tgc atg ata aca gac ttc ttc cct gaa gac att act gtg gag 480
Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu
      145          150          155          160
tgg cag tgg aat ggg cag cca gcg gag aac tac aag aac act cag ccc 528
Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro
      165          170          175
atc atg gac aca gat ggc tct tac ttc gtc tac agc aag ctc aat gtg 576
Ile Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val
      180          185          190
cag aag agc aac tgg gag gca gga aat act ttc acc tgc tct gtg tta 624
Gln Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu
      195          200          205
cat gag ggc ctg cac aac cac cat act gag aag agc ctc tcc cac tct 672
His Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser
      210          215          220

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cct ggt aaa tga
Pro Gly Lys
225

684

[0101]
SEQUENCE ID NO: 5
Length: 227
Type: amino acid
Topology: linear
Type of sequence: peptide
Sequence:

Val	Pro	Arg	Asp	Cys	Gly	Cys	Lys	Pro	Cys	Ile	Cys	Thr	Val	Pro	Glu
1				5				10					15		
Val	Ser	Ser	Val	Phe	Ile	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Val	Leu	Thr
			20				25						30		
Ile	Thr	Leu	Thr	Pro	Lys	Val	Thr	Cys	Val	Val	Val	Asp	Ile	Ser	Lys
		35				40						45			
Asp	Asp	Pro	Glu	Val	Gln	Phe	Ser	Trp	Phe	Val	Asp	Asp	Val	Glu	Val
	50					55					60				
His	Thr	Ala	Gln	Thr	Gln	Pro	Arg	Glu	Glu	Gln	Phe	Asn	Ser	Thr	Phe
	65				70					75				80	
Arg	Ser	Val	Ser	Glu	Leu	Pro	Ile	Met	His	Gln	Asp	Trp	Leu	Asn	Gly
			85					90						95	
Lys	Glu	Phe	Lys	Cys	Arg	Val	Asn	Ser	Ala	Ala	Phe	Pro	Ala	Pro	Ile
		100					105					110			
Glu	Lys	Thr	Ile	Ser	Lys	Thr	Lys	Gly	Arg	Pro	Lys	Ala	Pro	Gln	Val
	115					120					125				
Tyr	Thr	Ile	Pro	Pro	Pro	Lys	Glu	Gln	Met	Ala	Lys	Asp	Lys	Val	Ser
	130					135					140				
Leu	Thr	Cys	Met	Ile	Thr	Asp	Phe	Phe	Pro	Glu	Asp	Ile	Thr	Val	Glu
	145				150					155				160	
Trp	Gln	Trp	Asn	Gly	Gln	Pro	Ala	Glu	Asn	Tyr	Lys	Asn	Thr	Gln	Pro
			165					170					175		
Ile	Met	Asp	Thr	Asp	Gly	Ser	Tyr	Phe	Val	Tyr	Ser	Lys	Leu	Asn	Val
		180						185					190		
Gln	Lys	Ser	Asn	Trp	Glu	Ala	Gly	Asn	Thr	Phe	Thr	Cys	Ser	Val	Leu
	195						200					205			
His	Glu	Gly	Leu	His	Asn	His	His	Thr	Glu	Lys	Ser	Leu	Ser	His	Ser
	210				215						220				
Pro	Gly	Lys													
	225														

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[0102]

SEQUENCE ID NO: 6

Length: 1689

Type: nucleic acid

Number of chain: 2

Topology: linear

Type of sequence: other nucleic acid, Fusion DNA

Feature:

Name/key: CDS

Location: 1..1689

Method for determining the feature: S

Name/key: sig_peptide

Location: 1..48

Method for determining the feature: S

Name/Key: mat_peptide

Location: 49..1689

Method for determining the feature: S

Sequence:

atg cct ctc ctc ctc ttg ctg ctc ctg ctg cca agc ccc tta cac ccc	48
Met Pro Leu Leu Leu Leu Leu Leu Leu Pro Ser Pro Leu His Pro	
-16 -15 -10 -5	
cac ccc atc tgt gag gtc tcc aaa gtg gcc agc cac cta gaa gtg aac	96
His Pro Ile Cys Glu Val Ser Lys Val Ala Ser His Leu Glu Val Asn	
1 5 10 15	
tgt gac aag agg aat ctg aca gcg ctg cct cca gac ctg ccg aaa gac	144
Cys Asp Lys Arg Asn Leu Thr Ala Leu Pro Pro Asp Leu Pro Lys Asp	
20 25 30	
aca acc atc ctc cac ctg agt gag aac ctc ctg tac acc ttc tcc ctg	192
Thr Thr Ile Leu His Leu Ser Glu Asn Leu Leu Tyr Thr Phe Ser Leu	
35 40 45	
gca acc ctg atg cct tac act cgc ctc act cag ctg aac cta gat agg	240
Ala Thr Leu Met Pro Tyr Thr Arg Leu Thr Gln Leu Asn Leu Asp Arg	
50 55 60	
tgc gag ctc acc aag ctc cag gtc gat ggg acg ctg cca gtg ctg ggg	288
Cys Glu Leu Thr Lys Leu Gln Val Asp Gly Thr Leu Pro Val Leu Gly	
65 70 75 80	
acc ctg gat cta tcc cac aat cag ctg caa agc ctg ccc ttg cta ggg	336
Thr Leu Asp Leu Ser His Asn Gln Leu Gln Ser Leu Pro Leu Leu Gly	
85 90 95	

cag aca ctg cct gct ctc acc gtc ctg gac gtc tcc ttc aac cgg ctg	384
Gln Thr Leu Pro Ala Leu Thr Val Leu Asp Val Ser Phe Asn Arg Leu	
100 105 110	
acc tcg ctg cct ctt ggt gcc ctg cgt ggt ctt ggc gaa ctc caa gag	432
Thr Ser Leu Pro Leu Gly Ala Leu Arg Gly Leu Gly Glu Leu Gln Glu	
115 120 125	
ctc tac ctg aaa ggc aat gag ctg aag acc ctg ccc cca ggg ctc ctg	480
Leu Tyr Leu Lys Gly Asn Glu Leu Lys Thr Leu Pro Pro Gly Leu Leu	
130 135 140	
acg ccc aca ccc aag ctg gag aag ctc agt ctg gct aac aac aac ttg	528
Thr Pro Thr Pro Lys Leu Glu Lys Leu Ser Leu Ala Asn Asn Asn Leu	
145 150 155 160	
act gag ctc ccc gct ggg ctc ctg aat ggg ctg gag aat ctc gac acc	576
Thr Glu Leu Pro Ala Gly Leu Leu Asn Gly Leu Glu Asn Leu Asp Thr	
165 170 175	
ctt ctc ctc caa gag aac tcg ctg tat aca ata cca aag ggc ttt ttt	624
Leu Leu Leu Gln Glu Asn Ser Leu Tyr Thr Ile Pro Lys Gly Phe Phe	
180 185 190	
ggg tcc cac ctc ctg cct ttt gct ttt ctc cac ggg aac ccc tgg tta	672
Gly Ser His Leu Leu Pro Phe Ala Phe Leu His Gly Asn Pro Trp Leu	
195 200 205	
tgc aac tgt gag atc ctc tat ttt cgt cgc tgg ctg cag gac aat gct	720
Cys Asn Cys Glu Ile Leu Tyr Phe Arg Arg Trp Leu Gln Asp Asn Ala	
210 215 220	
gaa aat gtc tac gta tgg aag caa ggt gtg gac gtc aag gcc atg acc	768
Glu Asn Val Tyr Val Trp Lys Gln Gly Val Asp Val Lys Ala Met Thr	
225 230 235 240	
tct aac gtg gcc agt gtg cag tgt gac aat tca gac aag ttt ccc gtc	816
Ser Asn Val Ala Ser Val Gln Cys Asp Asn Ser Asp Lys Phe Pro Val	
245 250 255	
tac aaa tac cca gga aag ggg tgc ccc acc ctt ggt gat gaa ggt gac	864
Tyr Lys Tyr Pro Gly Lys Gly Cys Pro Thr Leu Gly Asp Glu Gly Asp	
260 265 270	
aca gac cta tat gat tac tac cca gaa gag gac act gag ggc gat aag	912
Thr Asp Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu Gly Asp Lys	
275 280 285	
gtg cgt gcc aca agg act gtg gtc aag ttc ccc acc aaa gcc cat aca	960
Val Arg Ala Thr Arg Thr Val Val Lys Phe Pro Thr Lys Ala His Thr	
290 295 300	
acc ccc tgg ggt cta ttc tac tca tgg tcc act gct tct cta gac gtg	1008
Thr Pro Trp Gly Leu Phe Tyr Ser Trp Ser Thr Ala Ser Leu Asp Val	
305 310 315 320	
ccc agg gat tgt ggt tgt aag cct tgc ata tgt aca gtc cca gaa gta	1056

Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val	
325 330 335	
tca tct gtc ttc atc ttc ccc cca aag ccc aag gat gtg ctc acc att	1104
Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile	
340 345 350	
act ctg act cct aag gtc acg tgt gtt gtg gta gac atc agc aag gat	1152
Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Lys Asp	
355 360 365	
gat ccc gag gtc cag ttc agc tgg ttt gta gat gat gtg gag gtg cac	1200
Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val His	
370 375 380	
aca gct cag acg caa ccc cgg gag gag cag ttc aac agc act ttc cgc	1248
Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg	
385 390 395 400	
tca gtc agt gaa ctt ccc atc atg cac cag gac tgg ctc aat ggc aag	1296
Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly Lys	
405 410 415	
gag ttc aaa tgc agg gta aac agt gca gct ttc cct gcc ccc atc gag	1344
Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile Glu	
420 425 430	
aaa acc atc tcc aaa acc aaa ggc aga ccg aag gct cca cag gtg tac	1392
Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr	
435 440 445	
acc att cca cct ccc aag gag cag atg gcc aag gat aaa gtc agt ctg	1440
Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser Leu	
450 455 460	
acc tgc atg ata aca gac ttc ttc cct gaa gac att act gtg gag tgg	1488
Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu Trp	
465 470 475 480	
cag tgg aat ggg cag cca gcg gag aac tac aag aac act cag ccc atc	1536
Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro Ile	
485 490 495	
atg gac aca gat ggc tct tac ttc gtc tac agc aag ctc aat gtg cag	1584
Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val Gln	
500 505 510	
aag agc aac tgg gag gca gga aat act ttc acc tgc tct gtg tta cat	1632
Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu His	
515 520 525	
gag ggc ctg cac aac cac cat act gag aag agc ctc tcc cac tct cct	1680
Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser Pro	
530 535 540	
ggt aaa tga	1689
Gly Lys	

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545

[0103]

SEQUENCE ID NO: 7

Length: 562

Type: amino acid

Topology: linear

Type of sequence: peptide

Sequence:

Met Pro Leu Leu Leu Leu Leu Leu Leu Leu Pro Ser Pro Leu His Pro
-16 -15 -10 -5
His Pro Ile Cys Glu Val Ser Lys Val Ala Ser His Leu Glu Val Asn
1 5 10 15
Cys Asp Lys Arg Asn Leu Thr Ala Leu Pro Pro Asp Leu Pro Lys Asp
20 25 30
Thr Thr Ile Leu His Leu Ser Glu Asn Leu Leu Tyr Thr Phe Ser Leu
35 40 45
Ala Thr Leu Met Pro Tyr Thr Arg Leu Thr Gln Leu Asn Leu Asp Arg
50 55 60
Cys Glu Leu Thr Lys Leu Gln Val Asp Gly Thr Leu Pro Val Leu Gly
65 70 75 80
Thr Leu Asp Leu Ser His Asn Gln Leu Gln Ser Leu Pro Leu Leu Gly
85 90 95
Gln Thr Leu Pro Ala Leu Thr Val Leu Asp Val Ser Phe Asn Arg Leu
100 105 110
Thr Ser Leu Pro Leu Gly Ala Leu Arg Gly Leu Gly Glu Leu Gln Glu
115 120 125
Leu Tyr Leu Lys Gly Asn Glu Leu Lys Thr Leu Pro Pro Gly Leu Leu
130 135 140
Thr Pro Thr Pro Lys Leu Glu Lys Leu Ser Leu Ala Asn Asn Asn Leu
145 150 155 160
Thr Glu Leu Pro Ala Gly Leu Leu Asn Gly Leu Glu Asn Leu Asp Thr
165 170 175
Leu Leu Leu Gln Glu Asn Ser Leu Tyr Thr Ile Pro Lys Gly Phe Phe
180 185 190
Gly Ser His Leu Leu Pro Phe Ala Phe Leu His Gly Asn Pro Trp Leu
195 200 205
Cys Asn Cys Glu Ile Leu Tyr Phe Arg Arg Trp Leu Gln Asp Asn Ala
210 215 220
Glu Asn Val Tyr Val Trp Lys Gln Gly Val Asp Val Lys Ala Met Thr
225 230 235 240
Ser Asn Val Ala Ser Val Gln Cys Asp Asn Ser Asp Lys Phe Pro Val

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                245                250                255
Tyr Lys Tyr Pro Gly Lys Gly Cys Pro Thr Leu Gly Asp Glu Gly Asp
                260                265                270
Thr Asp Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu Gly Asp Lys
                275                280                285
Val Arg Ala Thr Arg Thr Val Val Lys Phe Pro Thr Lys Ala His Thr
                290                295                300
Thr Pro Trp Gly Leu Phe Tyr Ser Trp Ser Thr Ala Ser Leu Asp Val
305                310                315                320
Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val
                325                330                335
Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile
                340                345                350
Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Lys Asp
                355                360                365
Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val His
370                375                380
Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg
385                390                395                400
Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly Lys
                405                410                415
Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile Glu
                420                425                430
Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr
                435                440                445
Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser Leu
450                455                460
Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu Trp
465                470                475                480
Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro Ile
                485                490                495
Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val Gln
                500                505                510
Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu His
                515                520                525
Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser Pro
530                535                540
Gly Lys
545
```

[0104]

SEQUENCE ID NO: 8

Length: 30

JPI0-113962

Type: nucleic acid

Number of chain: 1

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

agctaggatc cgagcccaga gggcccacaa

30

[0105]

SEQUENCE ID NO: 9

Length: 44

Type: nucleic acid

Number of chain: 1

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

cccaagcttc tcgagacata cctttcattt acccgagtc cggga

44

[0106]

SEQUENCE ID NO: 10

Length: 729

Type: nucleic acid

Number of chain: 2

Topology: linear

Type of sequence: cDNA to mRNA

Origine:

Organism: mouse

Type of cell: hybridoma cell strain W6/32

Feature:

Name/key: CDS

Location: 7..708

Method for determining the feature: S

Sequence:

ggatcc gag ccc aga ggg ccc aca atc aag ccc tgt cct cca tgc aaa 48

Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys

1

5

10

tgc cca gca cct aac ctc ttg ggt gga cca tcc gtc ttc atc ttc cct 96

Cys Pro Ala Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro

15

20

25

30

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cca aag atc aag gat gta ctc atg atc tcc ctg agc ccc ata gtc aca 144
Pro Lys Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr
      35              40              45

tgt gtg gtg gtg gat gtg agc gag gat gac cca gat gtc cag atc agc 192
Cys Val Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser
      50              55              60

tgg ttt gtg aac aac gtg gaa gta cac aca gct cag aca caa acc cat 240
Trp Phe Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His
      65              70              75

aga gag gat tac aac agt act ctc cgg gtg gtc agt gcc ctc ccc atc 288
Arg Glu Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile
      80              85              90

cag cac cag gac tgg atg agt ggc aag gag ttc aaa tgc aag gtc aac 336
Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn
      95              100             105             110

aac aaa gac ctg cca gcg ccc atc gag aga acc atc tca aaa ccc aaa 384
Asn Lys Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys
      115             120             125

ggg tca gta aga gct cca cag gta tat gtc ttg cct cca cca gaa gaa 432
Gly Ser Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu
      130             135             140

gag atg act aag aaa cag gtc act ctg acc tgc atg gtc aca gac ttc 480
Glu Met Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe
      145             150             155

atg cct gaa gac att tac gtg gag tgg acc aac aac ggg aaa aca gag 528
Met Pro Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu
      160             165             170

cta aac tac aag aac act gaa cca gtc ctg gac tct gat ggt tct tac 576
Leu Asn Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr
      175             180             185             190

ttc atg tac agc aag ctg aga gtg gaa aag aag aac tgg gtg gaa aga 624
Phe Met Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg
      195             200             205

aat agc tac tcc tgt tca gtg gtc cac gag ggt ctg cac aat cac cac 672
Asn Ser Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His
      210             215             220

acg act aag agc ttc tcc cgg act ccg ggt aaa tgaaaggat gtctcgagaa 725
Thr Thr Lys Ser Phe Ser Arg Thr Pro Gly Lys
      225             230

gctt 729

```

[0107]

SEQUENCE ID NO: 11

JP10-113962

Length: 233

Type: amino acid

Topology: linear

Type of sequence: peptide

Sequence:

Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro
1 5 10 15
Ala Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys
20 25 30
Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val
35 40 45
Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
50 55 60
Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
65 70 75 80
Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
85 90 95
Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
100 105 110
Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser
115 120 125
Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met
130 135 140
Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro
145 150 155 160
Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn
165 170 175
Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met
180 185 190
Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser
195 200 205
Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr
210 215 220
Lys Ser Phe Ser Arg Thr Pro Gly Lys
225 230

[0108]

SEQUENCE ID NO: 12

Length: 30

Type: nucleic acid

Number of chain: 1

JP10-113962

Topology: linear

Type of sequence: other nucleic acid, Artificial DNA

Sequence:

agctatctag acgagcccag agggcccaca

30

[0109]

SEQUENCE ID NO: 13

Length: 1707

Type: nucleic acid

Number of chain: 2

Topology: linear

Type of sequence: other nucleic acid, Fusion DNA

Feature:

Name/key: CDS

Location: 1..1707

Method for determining the feature: S

Name/key: sig_peptide

Location: 1..480

Method of determining the feature: S

Name/key: mat_peptide

Location: 49..1704

Method of determining the feature: S

Sequence:

atg cct ctc ctc ctc ttg ctg ctc ctg ctg cca agc ccc tta cac ccc	48
Met Pro Leu Leu Leu Leu Leu Leu Leu Pro Ser Pro Leu His Pro	
-16 -15	-10 -5
cac ccc atc tgt gag gtc tcc aaa gtg gcc agc cac cta gaa gtg aac	96
His Pro Ile Cys Glu Val Ser Lys Val Ala Ser His Leu Glu Val Asn	
1 5 10 15	
tgt gac aag agg aat ctg aca gcg ctg cct cca gac ctg ccg aaa gac	144
Cys Asp Lys Arg Asn Leu Thr Ala Leu Pro Pro Asp Leu Pro Lys Asp	
20 25 30	
aca acc atc ctc cac ctg agt gag aac ctc ctg tac acc ttc tcc ctg	192
Thr Thr Ile Leu His Leu Ser Glu Asn Leu Leu Tyr Thr Phe Ser Leu	
35 40 45	
gca acc ctg atg cct tac act cgc ctc act cag ctg aac cta gat agg	240
Ala Thr Leu Met Pro Tyr Thr Arg Leu Thr Gln Leu Asn Leu Asp Arg	

50	55	60	
tgc gag ctc acc aag ctc cag gtc gat ggg acg ctg cca gtg ctg ggg			288
Cys Glu Leu Thr Lys Leu Gln Val Asp Gly Thr Leu Pro Val Leu Gly			
65	70	75	80
acc ctg gat cta tcc cac aat cag ctg caa agc ctg ccc ttg cta ggg			336
Thr Leu Asp Leu Ser His Asn Gln Leu Gln Ser Leu Pro Leu Leu Gly			
85	90	95	
cag aca ctg cct gct ctc acc gtc ctg gac gtc tcc ttc aac cgg ctg			384
Gln Thr Leu Pro Ala Leu Thr Val Leu Asp Val Ser Phe Asn Arg Leu			
100	105	110	
acc tcg ctg cct ctt ggt gcc ctg cgt ggt ctt ggc gaa ctc caa gag			432
Thr Ser Leu Pro Leu Gly Ala Leu Arg Gly Leu Gly Glu Leu Gln Glu			
115	120	125	
ctc tac ctg aaa ggc aat gag ctg aag acc ctg ccc cca ggg ctc ctg			480
Leu Tyr Leu Lys Gly Asn Glu Leu Lys Thr Leu Pro Pro Gly Leu Leu			
130	135	140	
acg ccc aca ccc aag ctg gag aag ctc agt ctg gct aac aac aac ttg			528
Thr Pro Thr Pro Lys Leu Glu Lys Leu Ser Leu Ala Asn Asn Asn Leu			
145	150	155	160
act gag ctc ccc gct ggg ctc ctg aat ggg ctg gag aat ctc gac acc			576
Thr Glu Leu Pro Ala Gly Leu Leu Asn Gly Leu Glu Asn Leu Asp Thr			
165	170	175	
ctt ctc ctc caa gag aac tcg ctg tat aca ata cca aag ggc ttt ttt			624
Leu Leu Leu Gln Glu Asn Ser Leu Tyr Thr Ile Pro Lys Gly Phe Phe			
180	185	190	
ggg tcc cac ctc ctg cct ttt gct ttt ctc cac ggg aac ccc tgg tta			672
Gly Ser His Leu Leu Pro Phe Ala Phe Leu His Gly Asn Pro Trp Leu			
195	200	205	
tgc aac tgt gag atc ctc tat ttt cgt cgc tgg ctg cag gac aat gct			720
Cys Asn Cys Glu Ile Leu Tyr Phe Arg Arg Trp Leu Gln Asp Asn Ala			
210	215	220	
gaa aat gtc tac gta tgg aag caa ggt gtg gac gtc aag gcc atg acc			768
Glu Asn Val Tyr Val Trp Lys Gln Gly Val Asp Val Lys Ala Met Thr			
225	230	235	240
tct aac glg gcc agt gtg cag tgt gac aat tca gac aag ttt ccc gtc			816
Ser Asn Val Ala Ser Val Gln Cys Asp Asn Ser Asp Lys Phe Pro Val			
245	250	255	
tac aaa tac cca gga aag ggg tgc ccc acc ctt ggt gat gaa ggt gac			864
Tyr Lys Tyr Pro Gly Lys Gly Cys Pro Thr Leu Gly Asp Glu Gly Asp			
260	265	270	
aca gac cta tat gat tac tac cca gaa gag gac act gag ggc gat aag			912
Thr Asp Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu Gly Asp Lys			
275	280	285	

gtg cgt gcc aca agg act gtg gtc aag ttc ccc acc aaa gcc cat aca	960
Val Arg Ala Thr Arg Thr Val Val Lys Phe Pro Thr Lys Ala His Thr	
290 295 300	
acc ccc tgg ggt cta ttc tac tca tgg tcc act gct tct cta gac gag	1008
Thr Pro Trp Gly Leu Phe Tyr Ser Trp Ser Thr Ala Ser Leu Asp Glu	
305 310 315 320	
ccc aga ggg ccc aca atc aag ccc tgt cct cca tgc aaa tgc cca gca	1056
Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro Ala	
325 330 335	
cct aac ctc ttg ggt gga cca tcc gtc ttc atc ttc cct cca aag atc	1104
Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Ile	
340 345 350	
aag gat gta ctc atg atc tcc ctg agc ccc ata gtc aca tgt gtg gtg	1152
Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val Val	
355 360 365	
gtg gat gtg agc gag gat gac cca gat gtc cag atc agc tgg ttt gtg	1200
Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe Val	
370 375 380	
aac aac gtg gaa gla cac aca gct cag aca caa acc cat aga gag gat	1248
Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp	
385 390 395 400	
tac aac agt act ctc cgg gtg gtc agt gcc ctc ccc atc cag cac cag	1296
Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His Gln	
405 410 415	
gac tgg atg agt ggc aag gag ttc aaa tgc aag gtc aac aac aaa gac	1344
Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys Asp	
420 425 430	
ctg cca gcg ccc atc gag aga acc atc tca aaa ccc aaa ggg tca gta	1392
Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser Val	
435 440 445	
aga gct cca cag gta tat gtc ttg cct cca cca gaa gaa gag atg act	1440
Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met Thr	
450 455 460	
aag aaa cag gtc act ctg acc tgc atg gtc aca gac ttc atg cct gaa	1488
Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro Glu	
465 470 475 480	
gac att tac gtg gag tgg acc aac aac ggg aaa aca gag cta aac tac	1536
Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn Tyr	
485 490 495	
aag aac act gaa cca gtc ctg gac tct gat ggt tct tac ttc atg tac	1584
Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met Tyr	
500 505 510	
agc aag ctg aga gtg gaa aag aag aac tgg gtg gaa aga aat agc tac	1632

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```
Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser Tyr
      515                      520                      525
tcc tgt tca gtg gtc cac gag ggt ctg cac aat cac cac acg act aag 1680
Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr Lys
      530                      535                      540
agc ttc tcc cgg act ccg ggt aaa tga 1707
Ser Phe Ser Arg Thr Pro Gly Lys
545                      550
```

[0110]

SEQUENCE ID NO: 14

Length: 568

Type: amino acid

Topology: linear

Type of sequence: peptide

Sequence:

```
Met Pro Leu Leu Leu Leu Leu Leu Leu Pro Ser Pro Leu His Pro
-16 -15                      -10                      -5
His Pro Ile Cys Glu Val Ser Lys Val Ala Ser His Leu Glu Val Asn
  1                      5                      10                      15
Cys Asp Lys Arg Asn Leu Thr Ala Leu Pro Pro Asp Leu Pro Lys Asp
      20                      25                      30
Thr Thr Ile Leu His Leu Ser Glu Asn Leu Leu Tyr Thr Phe Ser Leu
      35                      40                      45
Ala Thr Leu Met Pro Tyr Thr Arg Leu Thr Gln Leu Asn Leu Asp Arg
  50                      55                      60
Cys Glu Leu Thr Lys Leu Gln Val Asp Gly Thr Leu Pro Val Leu Gly
  65                      70                      75                      80
Thr Leu Asp Leu Ser His Asn Gln Leu Gln Ser Leu Pro Leu Leu Gly
      85                      90                      95
Gln Thr Leu Pro Ala Leu Thr Val Leu Asp Val Ser Phe Asn Arg Leu
      100                      105                      110
Thr Ser Leu Pro Leu Gly Ala Leu Arg Gly Leu Gly Glu Leu Gln Glu
      115                      120                      125
Leu Tyr Leu Lys Gly Asn Glu Leu Lys Thr Leu Pro Pro Gly Leu Leu
      130                      135                      140
Thr Pro Thr Pro Lys Leu Glu Lys Leu Ser Leu Ala Asn Asn Asn Leu
      145                      150                      155                      160
Thr Glu Leu Pro Ala Gly Leu Leu Asn Gly Leu Glu Asn Leu Asp Thr
      165                      170                      175
Leu Leu Leu Gln Glu Asn Ser Leu Tyr Thr Ile Pro Lys Gly Phe Phe
      180                      185                      190
```

Gly Ser His Leu Leu Pro Phe Ala Phe Leu His Gly Asn Pro Trp Leu
 195 200 205
 Cys Asn Cys Glu Ile Leu Tyr Phe Arg Arg Trp Leu Gln Asp Asn Ala
 210 215 220
 Glu Asn Val Tyr Val Trp Lys Gln Gly Val Asp Val Lys Ala Met Thr
 225 230 235 240
 Ser Asn Val Ala Ser Val Gln Cys Asp Asn Ser Asp Lys Phe Pro Val
 245 250 255
 Tyr Lys Tyr Pro Gly Lys Gly Cys Pro Thr Leu Gly Asp Glu Gly Asp
 260 265 270
 Thr Asp Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu Gly Asp Lys
 275 280 285
 Val Arg Ala Thr Arg Thr Val Val Lys Phe Pro Thr Lys Ala His Thr
 290 295 300
 Thr Pro Trp Gly Leu Phe Tyr Ser Trp Ser Thr Ala Ser Leu Asp Glu
 305 310 315 320
 Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro Ala
 325 330 335
 Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Ile
 340 345 350
 Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val Val
 355 360 365
 Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe Val
 370 375 380
 Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp
 385 390 395 400
 Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His Gln
 405 410 415
 Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys Asp
 420 425 430
 Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser Val
 435 440 445
 Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met Thr
 450 455 460
 Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro Glu
 465 470 475 480
 Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn Tyr
 485 490 495
 Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met Tyr
 500 505 510
 Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser Tyr
 515 520 525
 Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr Lys

530	535	540
Ser Phe Ser Arg Thr Pro Gly Lys		
545	550	

[Brief Explanation of the Drawings]

[Fig. 1] outlines the construction of GPIb-mIgG1Fc expression system.

[Fig. 2] outlines the construction of GPIb-mIgG2aFc expression system.

[Fig. 3] shows binding amount of immobilized von Willebrand factor and a chimeric protein plotted against amount of botrocetin (ELISA).

[Fig. 4] shows activity of inhibition substance for the binding of von Willebrand factor and a chimeric protein (ELISA).

[Fig. 5] shows activity of inhibition substance for the binding of von Willebrand factor and a chimeric protein (Eu-labeling method).

[Fig. 6] shows exemplary quantification of glyocalicin in human plasma (ELISA).

[Fig. 7] shows exemplary quantification of glyocalicin in human plasma (Eu-labeling method).

[Name of document] Abstract

[Summary]

[Problem]

A method for conveniently detecting binding between the von Willebrand factor and glycoprotein Ib and a means to be used therein.

[Means to solve the problem]

The von Willebrand factor fixed in a reactor immobilized in a reaction vessel in the presence of botrocetin is bound to a chimeric protein constructed by fusing the carboxyl terminal of a partial protein containing the von Willebrand factor-binding site of glycoprotein Ib with the amino terminal of the Fc region of an immunoglobulin molecule. Then the Fc region of the above immunoglobulin molecule is detected to thereby detect the binding between the von Willebrand factor and the glycoprotein Ib or inhibition of this binding.

[Selected drawing] Fig. 4